Midline1 and the development of the cranial peripheral nervous system

Conference or Workshop Item

Midline1 and the Development of the Cranial Peripheral Nervous System

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Introduction

- Genetic abnormalities involving the skull and facial region account for around 1/3 of birth defects
- Opitz BBB/G syndrome is a disorder that gives rise to craniofacial malformations, such as cleft lip/palate, other symptoms include mental retardation, hypertelorism, gastrointestinal defects and other midline defects
- Patients with X-linked Opitz BBB/G Syndrome have loss of function mutations in the gene Midline 1 (Mid1)
- Mid1 functions as a ubiquitin ligase, targeting Protein Phosphatase 2A (PP2A) for degradation
- Mid1 also binds to, and can form protein complexes on, microtubules

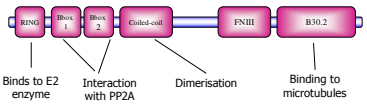

Figure 1: A schematic diagram of the protein structure of Mid1 illustrating key functional domains

Methods

- Chick embryos between 6 - 25ss were used as a model for cranial development
- ISH were performed using Mid1 and Sox10 probes [1,2]
- A Mid1 IRES GFP expression construct (pCAB.Mid1) and a Dominant Negative Mid1 IRES GFP construct (pCAB.DN-Mid1) were used to alter Mid1 activity levels [1]
- A PP2A expression construct (pCAB.PP2A) was used to increase the protein levels of PP2A in electroporated cells
- A GPF only expressing construct was used as a control (pCAB.GFP) [1]
- The expression constructs were injected into the lumen of the neural tube and unilaterally electroporated into the right side of the hindbrain neural tube.
- Okadaic acid (OA) was used to inhibit PP2A activity. For *in vitro* inhibition, the OA was diluted in the culture media to 1nM.

Expression Pattern of Mid1

- Embryos were processed by in-situ hybridisation (ISH) for Mid1 and Sox10 (neural crest cell marker)
- The results showed that at the 13 somite stage (ss) Mid1 is strongly expressed in rhombomere 2 and the mesenchyme adjacent to r1-r2 and the midbrain
- Sox10 staining showed the mesenchymal Mid1 staining overlaps with the Sox10
- Transverse sections through these embryos showed that the Sox10-Mid1 expression does overlap close to the neural tube, therefore implying that r1/r2 NCC's express Mid1, but as the neural crest migrates into the branchial arch it down-regulates Mid1 expression.

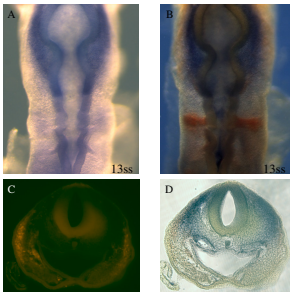

Figure 2: Insitu hybridisation on 13 somite stage chick embryos. Mid1 (Blue), Sox10 (Orange/Red) on wholemount embryos (**A,B**) and transverse sections through r1 (**C,D**). Fluorescence illumination in (**C**) is used to enhance the sensitivity of Fast Red-stained Sox10 mRNA. The fluorescence signal is masked in cells that co-stain with BM Purple for Mid1 mRNA..

Knockdown of Endogenous Mid1

- Chick embryos were electroporated with a Dominant Negative – Mid1 (DN-Mid1) expression construct at 8ss and incubated to 23ss
- Neurofilament staining for neurons showed a reduction in the size of the trigeminal ganglia.

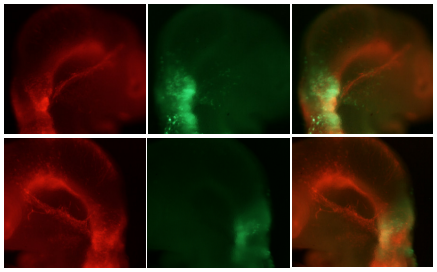

Figure 3: An embryo electroporated unilaterally, targeting rhombomere 2 with a DN-Mid1 expressing construct at 8ss and then incubated to 23ss. Embryo was stained for neurofilament (red) and GFP (green).

Over-expression of PP2A in r2

- One function of Mid1 is to target PP2A for destruction. Therefore to determine whether excess PP2A could explain the DN-Mid1 phenotype, we over expressed PP2A in r2.
- Embryos were electroporated unilaterally into rhombomere 2 at 8ss and incubated to 23ss
- Over-expression of PP2A in r2 gave the same ganglia phenotype as the DN-Mid1 construct, therefore implying that Mid1 is acting through its PP2A ubiquitination function to affect the development of the ganglia

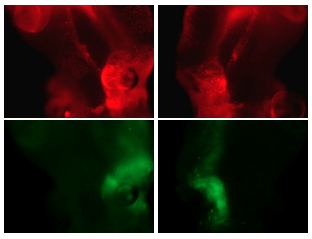

Figure 4: An embryo electroporated unilaterally targeting rhombomere 2 with a PP2A expressing construct at 8ss incubated to 23ss. Embryo was stained for Neurofilament (red) and GFP (green).

Ectopic Expression of Mid1

- To investigate if Mid1 could promote gangliogenesis in a neural crest population that does not normally express Mid1, embryos were electroporated in r4 with a Mid1 expressing construct at 10ss and incubated to 25ss
- The expression of Mid1 in r4 and r4 NCC's resulted in premature development of the facial-acoustic ganglia
- These results further support the theory that Mid1 has a role in the development of the cranial ganglia

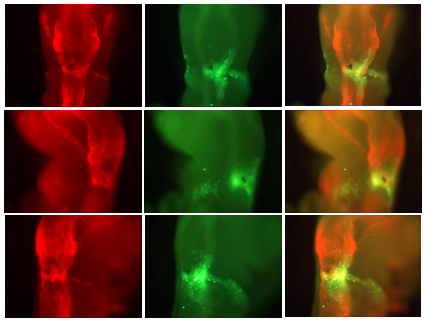

Figure 5: An embryo electroporated unilaterally targeting rhombomere 4 with a Mid1 expressing construct at 10ss incubated to 25ss. Embryo was stained for Neurofilament (red) and GFP (green).

PP2A inhibition *In Vitro*

- Okadaic Acid (OA) is an inhibitor of PP2A. Therefore exposure of r4 cells to OA should mimic the effect of Mid1 expression. When the r4 NCC's are exposed to OA, the same Mid1 ganglia phenotype is observed.
- Cranial neural crest cells from rhombomere 4 were grown in culture with Okadaic Acid (OA) and time-lapsed for 8 hours
- The speed of the cells were measured using the MtrackJ application in ImageJ.
- The results show that on addition of OA the r4 cNCC's speed up significantly in the first 2 hours of culture and the speed remains high for the rest of the culture period. In comparison, when r2 cNCC's are cultured with OA there is no significant difference in the speed of the cells compared to untreated control cells.

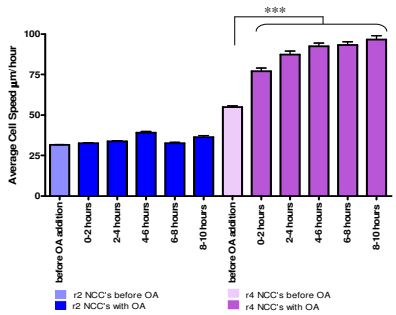

Figure 6: Graph showing speed of r2 and r4 cranial neural crest cells before and after the addition of Okadaic acid (1nM) in the cell culture media. P<0.001 (***)

Mid1 and Neural Crest Cell Delamination

- In order to investigate if Mid1 was affecting NCC delamination from the neural tube embryos were electroporated with the Mid1 and GFP constructs and stained for Pax3, a neural crest marker.
- Confocal scans of transverse sections through r4 show that there are fewer NCC's on the Mid1 electroporated side of the neural tube, which is not seen on the GFP electroporated sections. No differences were detected in cell death or birth within the neural tube or the crest streams (not shown).
- Taken in conjunction with our data on neural crest speed, it would appear that Mid1 acts to promote delamination of NCC's from the neural tube'

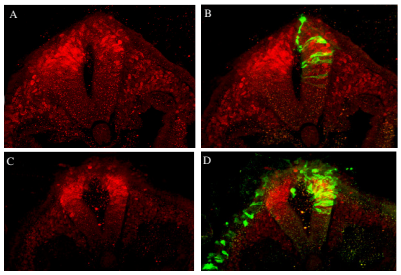

Figure 7: Neural crest cell staining using Pax3 on sections through r4. Pax3 (red) and GFP (green). Figure shows sections through r4 of embryos electroporated with Mid1 (**A,B**) and the GFP control construct (**C,D**)

Conclusions

We show here evidence that Mid1 is involved in the development of the cranial ganglia and that prevention of endogenous Mid1 activity leads to stunted ganglia development, whereas ectopic expression of Mid1 leads to premature ganglia development.

We also show that PP2A is the most likely mechanism through which Mid1 is affecting ganglia development, possibly through affecting neural crest cell speed and promoting delamination of neural crest cells from the neural tube.

Acknowledgements

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References:

1. Granata, A et al. *Developmental Biology.* 258(2) 397-405 (2003)
2. Cheng, Y., et al., *Brain Res Dev Brain Res.* 121(2): p. 233-41 (2000)